

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1 AGENCY USE ONLY (Leave blank)		2 REPORT DATE Nov 1990		3 REPORT TYPE AND DATES COVERED Final 1 Jul 87 - 14 Sep 90	
4 TITLE AND SUBTITLE Molecular Architecture for Reagentless Immunosensors				5 FUNDING NUMBERS DAAL03-87-K-0129	
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Michigan Ann Arbor, MI 48104				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING MONITORING AGENCY NAME(S) AND ADDRESS(ES) U. S. Army Research Office P. O. Box 12211 Research Triangle Park, NC 27709-2211				10. SPONSORING MONITORING AGENCY REPORT NUMBER ARO 24665.9-LS	
11. SUPPLEMENTARY NOTES The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.					
12a. DISTRIBUTION AVAILABILITY STATEMENT Approved for public release; distribution unlimited.				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) In this project, work focused on three major methodologies to simplify the performance of solid-phase immunoassays: 1. Immobilization of immunoglobulins on solid matrices by maintaining the specific binding activity of the idiotypic sites; 2. separation of bound from free antigen without user's intervention; and 3. signal generation as a quantitative indicator of analyte concentration in samples. The results of these investigations are documented in detail in the publications and summarized in the final report.					
14. SUBJECT TERMS Immunoglobulins, Innunosensors, Immunoassays, Proteins, Biosensors				15. NUMBER OF PAGES 6	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UL		

AD-A231 092

FINAL REPORT

1. ARO PROPOSAL NUMBER: 24665-LS
2. PERIOD COVERED BY REPORT: 1 July 1987 -14 September 1990
3. TITLE OF PROPOSAL: Molecular Architecture for Reagentless Immunosensors
4. CONTRACT OR GRANT NUMBER: DAAL03-87-K-0129
5. NAME OF INSTITUTION: University of Michigan
6. AUTHORS OF REPORT: Willfried Schramm and Richard G. Lawton
7. LIST OF MANUSCRIPTS SUBMITTED OR PUBLISHED UNDER ARO SPONSORSHIP DURING THIS REPORTING PERIOD, INCLUDING JOURNAL REFERENCES:

DEVELOPMENT OF A NOVEL PHOTOREACTIVE CALMODULIN DERIVATIVE: CROSS-LINKING OF PURIFIED ADENYLATE CYCLASE FROM BOVINE BRAIN. JK Harrison, RG Lawton, ME Gnegy. *Biochemistry* 28:6023-6027, 1989.

P-NITROPHENYL 3-DIAZOPYRUVATE AND DIAZOPYRUVAMIDES. A NEW FAMILY OF PHOTOREACTIVATABLE CROSS-LINKING BIOPROBES. VS Goodfellow, M Settineri, RG Lawton. *Biochemistry* 28:6346-6360, 1989.

BIOANALYTICAL DETECTION SYSTEM. W. Schramm. Patent pending.

CONTINUOUS MONITORING OF ANALYTE CONCENTRATIONS. W. Schramm and S-H. Paek. First World Congress on Biosensors, Singapore, May 1990. *Biosensors Bioelectron.*, submitted 1990.

MODELING OF IMMUNOSENSORS UNDER NON-EQUILIBRIUM CONDITIONS. PART I: MATHEMATIC MODELING OF PERFORMANCE CHARACTERISTICS. W. Schramm and S-H. Paek. Submitted 1990.

MODELING OF IMMUNOSENSORS UNDER NON-EQUILIBRIUM CONDITIONS. PART II: EXPERIMENTAL DETERMINATION OF PERFORMANCE CHARACTERISTICS. W. Schramm and S-H. Paek. Submitted 1990.

ENZYME-ANALYTE CONJUGATES AS SIGNAL GENERATORS FOR AMPEROMETRIC IMMUNOSENSORS: IMMUNOCHEMICAL PHENOMENA RELATED TO THE DETECTION OF HAPTEN MOLECULES. W. Schramm.

S-H. Paek, and T. Yang. In: Biosensor Technology. Fundamentals and Applications. Eds.: Buck, R.P., Hatfield, W.E., Umana, M., Bowden, E.F.: Marcel Dekker, New York 1990.

IRREVERSIBLE ENZYME-SHUTTLE IMMUNOASSAY. S-H. Paek and W. Schramm. *Anal. Chem.*, submitted 1990.

ENHANCED PERFORMANCE OF IMMUNOASSAYS WITH DEFINED ANALYTE-ENZYME CONJUGATES AS SIGNAL GENERATORS. S-H Paek, L.G. Bachas, and W. Schramm. *Anal. Chem.* Submitted 1990

DIFFERENT RECOGNITION OF IMMOBILIZED IMMUNOGLOBULINS BY RADIOLABELED AND ENZYME LABELED ANTIGEN DERIVATIVES. W. Schramm and S-H. Paek. In preparation.

8. SCIENTIFIC PERSONNEL SUPPORTED BY THIS PROJECT AND DEGREES AWARDED DURING THIS REPORTING PERIOD:

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DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
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Availability Codes	
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The overall objective of this project was to investigate new approaches for the construction of sensors that are based on specific binding proteins (immunoglobulins) for analyte recognition. The methods that use immunoglobulins for the quantitative determination of molecules in aqueous solutions traditionally require several processing steps, many of them are very unfamiliar to untrained persons. Therefore, almost all of these tests cannot be performed outside the laboratory. It was our goal to reduce the number of steps required to process samples. To do so, the operations that are involved in an immunoassay, have to be built into the device so that they are automatically performed and become non-obvious to the user. Ideally, the number of steps performed by the user are reduced to bringing the sample into contact with a device and read out the result (Figure 1).

IMMUNOASSAYS

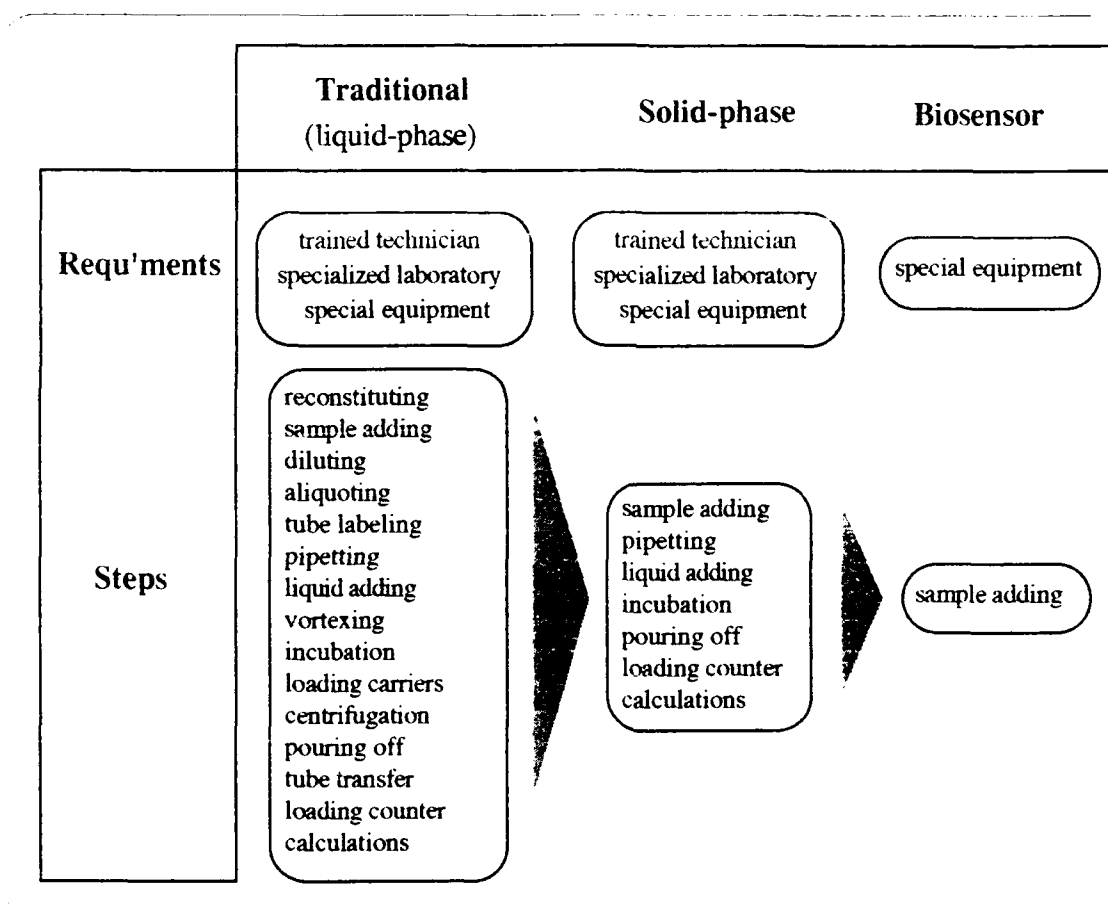


Figure 1. The number of steps involved in a traditional immunoassay are substantially reduced in solid-phase assays. Ideally, biosensors incorporate many of the operation steps in a device and reduce the complexity further such that non-trained persons can perform an analysis of samples.

In this project, we focused on three major methodologies to simplify the performance of solid-phase immunoassays:

1. Immobilization of immunoglobulins on solid matrices by maintaining the specific binding activity of the idiotypic sites;

2. separation of bound from free antigen without user's intervention; and
3. signal generation as a quantitative indicator of analyte concentration in samples.

The results of these investigations are documented in detail in the publications listed above and will be summarized in the following.

Immobilization of immunoglobulins

Immunoglobulins are comparatively stable proteins that maintain their ability to specifically form binding complexes with antigens even after desiccation and reconstitution in aqueous media. However, if precipitated on solid surfaces, the proteins can bind very tightly to the matrix and the ability to bind antigens can be severely impaired. The binding energy depends on the type of the surface. Not only the chemical structure determines the orientation and the quality of surface interaction, but also the physical history of solid matrices (e.g., number of radicals on the surface, degree of hydration, deposits of airborne molecules, physical structure). Since typically only monomolecular layers of immunoglobulins are deposited on solid matrices, variation in surface properties can severely affect the number of immunoglobulin molecules deposited and their immunochemical properties. Therefore, we modified solid matrices by treating the surface with polymers. To these polymers, we chemically bound the immunoglobulins.

Several chemical reactions have been investigated for the modification of immunoglobulins and subsequent immobilization. The objectives for designing schemes for the activation of immunoglobulins were:

- Chemical reaction in aqueous media;
- no crosslinking of domains of the protein structure of immunoglobulins;
- maintaining the integrity of the idiotypic sites of immunoglobulins preferentially by activating the Fc region of the molecule;
- introducing linker molecules of defined length;
- selecting linker molecules that do not increase non-specific binding of analyte molecules.

For parts of this project, immobilization of a heterobifunctional immunoglobulin with the ability to freely rotate around the anchorage point was required. Although the original concept using an immunoglobulin that recognizes different antigens with its two idiotypic sites was changed during the course of the experiments, the methods for introducing linker molecules of considerable length between an antibody and the solid matrix proved to be very useful for the development of analytical systems that use analyte-enzyme conjugates as signal generators for the analysis of small molecules. Since an analyte-enzyme conjugate is usually substantially larger than the native analyte to be detected (typically 100-times larger) but both should be able to bind in competitive immunoassays, the bulkier conjugate may be sterically hindered to bind to a rigidly immobilized immunoglobulin. We found that linkers between the immunoglobulin and the surface that provide flexibility for rotation to the antibody exhibit linear correlations in Scatchard analyses with analyte-enzyme conjugates, suggesting the presence of a single population of binding sites for the conjugate.

Another concern in maintaining the integrity of antibody binding sites is that the molecules do not recede towards the surface of the solid matrix, even if the matrix is modified and the immunoglobulins are attached by chemical links to the surface. We have investigated the

modification of matrices with molecules that provide different charges to the surface, and that support the immunoglobulins such that they cannot collapse towards the surface.

Separation of bound from free antigen

Most immunoassays require a separation step that removes free antigens (native and tracer) from that bound to antibodies (heterogeneous immunoassays). Homogeneous assays are usually much less sensitive and have, therefore, limited applications. In particular the quantitative estimation of small molecules in concentrations $<10^{-6}$ mol/L does not leave much choice but the technique of competitive immunoassay (as opposed to sandwich assays for larger molecules) which is a method that requires separation.

The separation step is a serious limitation for the development of simple immunotests with a minimal number of handling steps. Therefore, we had originally investigated a method that achieved separation at the molecular level by quenching a fluorescent molecule as signal generator. Since the signal yield in this method proved to be too low to be monitored with field-use instrumentation, we changed the original concept for separation of bound from free antigen.

In the current concept, we use immunochromatography for the separation step. With this method, a competitive immunoassay can be performed on a test strip and the separation step is not left to action by the user but built into the device by using capillary action to remove unbound antigen from the immobilized antibody. This method has the advantage that it is more versatile in making use of existing techniques signal generation (i.e., by using analyte-enzyme conjugates that generate colored products or electrochemically active molecules).

Signal generation

Since binding of analytes to antibodies can usually not be detected directly by physical means, some method has to be devised to obtain a signal in response to binding of analyte from a sample to immunoglobulins. The original approach studied in this project was to use fluorescein immobilized on a matrix. The fluorescein would be quenched upon binding to an antibody. The system was set up such that binding to fluorescein occurred proportional to the concentration of analyte in a sample. The key reagent for this assay is a heterobifunctional binder, an antibody that binds with one idiotype sites to the analyte, and with the other site to fluorescein. During the course of the experiments, it became clear that a laser source would be required to generate large enough a signal for the number of fluorescent molecules that could be immobilized on the surface of solid matrices. Since this would have been a major limitation for simple field-use instruments, we modified the concept and used instead a heterobifunctional conjugate in connection with two antibodies (see progress reports). This experimental model, in combination with immunochromatography, substantially simplified the development of immunotests.

The theoretical basis of this dual-antibody system that provides two signals for each measurement has been developed and confirmed by experiments (see attached manuscript). We have used enzymes for the development of colored products as signal generators for the studies in model systems. However, we also have initiated a program to extend this concept for the electrochemical detection of signals generated by enzymes. It is planned to incorporate the immunotest strip into a disposable electrode that will be attached to a hand-

held potentiometer. Instrumentation will thus be kept inexpensive and simple to handle while potentially subjective color read-out will be avoided.

Outlook

The basic concept of the double-antibody assay based on immunochromatography has been demonstrated. As a result of this project, several technologies have been developed that, if incorporated into immunotest strips, can be operated outside the laboratory by non-trained personnel. This technology will be further developed in two stages for which financial support from different sources is currently being sought. In Phase I, a pre-manufacture prototype immunotest strip with particular emphasis on electrochemical signal detection, will be developed. Phase II will be mainly devoted to manufacture development and to clinical trials. Initially, the test will be adapted for the determination of the cocaine metabolite benzoylecgonine. The suitability of the test strip for the measurement of the cocaine derivative in saliva will be studied. The concept will then be expanded to other drugs of abuse and to therapeutic drugs.

Acknowledgement

We are grateful for the support of the Army Research Office for this project. The excellent communication between the groups working in the areas of Bioanalytic and Biotechnology and the Army Research Office is greatly appreciated. Special thanks to Dr. Shirley R. Tove for her role as facilitator, organizer, and advisor.